Exhibit B

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Conformational changes in rodent and human α-fetoprotein: influence of fatty acids

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Binding, spectral and immunological studies were performed to demonstrate the conformational changes in rodent and human α-fetoprotein (AFP) induced by a free fatty acid environment. Scatchard analysis of estradiol (E2) binding to purified rat AFP indicated that unsaturated fatty acids changed the number of binding E2 sites and the apparent E2 equilibrium dissociation constant which varied non-linearly with docosahexaenoic acid concentration. UV spectral analysis of rodent and human AFPs showed that the absorbance minimum of AFP incubated with unsaturated fatty acid (L-AFP) was red-shifted, broadened and less pronounced than that of purified native AFP (N-AFP). Immunochemical studies with specific polyclonal antibodies to purified rodent and human AFPs (N-AFP antibodies) showed that these proteins lost immunoreactivity after incubation with unsaturated fatty acid. N-AFP antibodies recognized fewer epitopes on L-AFP than on N-AFP, whatever the species. Specific anti-rat L-AFP antibodies were used to demonstrate specific epitopes on rat L-AFP. Rat L-AFP antibodies did not recognize rat N-AFP. Saturated fatty acids were without effect on the binding, spectral and immunological properties of rodent and human AFPs. RIA or ELISA values for human AFP from fetal serum, hepatoma serum, and cord serum, were reduced 80, 50 and 5%, respectively, by unsaturated fatty acids. This decrease correlated with the relative percentage of polyunsaturated fatty acid in each biological fluid. Such results indicate that an unsaturated fatty acid environment induces conformational changes in AFP which may modulate the endocrine and immune functions of this protein.

Introduction

Mammalian α-feto-proteins (AFPs) are oncofetal antigens which exhibit a complex molecular heterogeneity involving differences in size, charge, carbohydrate moiety and ligand contents (fatty acids and estrogens) [1–3]. The molecular polymorphism of AFPs is associated with considerable intra- and inter-species functional heterogeneity reflected in its binding [1–3] and immunomodulatory properties [4].

While all mammalian AFPs bind non-esterified fatty acids (NEFAs), especially unsaturated fatty acids (UFA), with high affinity ($K_a = 10^6$–10^7 M^{-1}) [5–8], only rodent AFPs have been shown to bind estrogen [9]. Several studies have shown that the binding of estrogen to rodent AFP is modulated by the endogenous NEFA content of the protein, which in turn depends on its exogenous NEFA environment [5–6,10]. Thus, UFA can inhibit estrogen binding to both mouse and rat AFP. Inhibition is dose-dependent and varies with the degree of fatty acid unsaturation, polyunsaturated fatty acids such as arachidonic and docosahexaenoic acids are the most efficient. Fatty acids (FA) have been reported to have similar effects on the binding properties of other plasma proteins, such as sex steroid-binding protein (SBP) [11] corticosteroid-binding globulin (CBG) [12], and on the binding of thyroid hormones by plasma proteins [13].

The immunoregulatory effects of AFP include the suppression of primary and secondary antibody responses [14], lymphocyte transformation induced by mitogens and allogenic cells [15,16], and Ia macrophage antigen expression [17]. However, other authors were unable to obtain similar results using purified AFP

Abbreviations: AFP, α-fetoprotein; E2, estradiol; E1, estrone; L-AFP, AFP incubated with unsaturated fatty acid; N-AFP, native AFP; NEFA, nonesterified fatty acid; UFA, unsaturated fatty acid; FA, fatty acids; SBP, sex steroid-binding protein; CBG, corticosteroid-binding protein; PBS, phosphate-buffered saline.

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preparations. Some obtained either stimulatory effects or no response [18,19]. The varying immunomodulatory activities of AFP could be related to the use of different forms of AFP, e.g., AFP isoforms (variable carbohydrate structure) and/or AFP holoforms which may vary according to their variable ligand contents (NEFAs and estrogens) which in turn will depend on the biological source of AFP and the purification method used [20–23].

The relationship between the NEFA-dependent biological activity of AFP and NEFA-induced conformational changes was examined by analysing the physicochemical properties of rat, mouse and human AFPs in the presence of saturated or unsaturated fatty acids. The three parameters studied were the NEFA-induced changes in the E2 binding properties of purified rat AFP, the UV spectral properties of rat and human AFP, and the serological reactivity of mammalian AFP. Special attention was paid to the immunological behaviour of mammalian AFP towards antibodies directed against native (N-AFP) or fatty acid-loaded AFP (L-AFP) and on the differences in human AFP immunological reactivity produced by the lipid environment of various biological fluids (embryo serum, hepatoma serum and cord serum). The results indicate that unsaturated fatty acids induce significant changes in the functional properties, e.g., binding and immunoreactivity of AFP, which are associated with conformational changes in the structure of AFP itself.

Materials and Methods

Chemicals

[6,7-^{3}H]Estradiol (41 Ci/mmol) purchased from Amersham International Ltd. was 98–99% pure and was regularly tested to ensure that level of purity.

Unlabeled compounds

Estradiol (E2) and estrone (El) were supplied by Roussel-Uclaf Research Centre (Romainville, France). The following saturated and unsaturated fatty acids were purchased from Sigma: tetradecanoic acid (14 : 0, myristic acid), hexadecanoic acid (16 : 0, palmitic acid), *cis*-7-hexadecanoic acid (16 : 1, palmitoleic acid), *cis*-9-octadecanoic acid (18 : 1, oleic acid), 9,12-octadecadienoic acid (18 : 2, linoleic acid), 6,9,12-octadecatrienoic acid (18 : 3, linolenic acid), 5,8,11,14-eicosatetraenoic acid (20 : 4, arachidonic acid), 4,7,10,13,16, 19-docosahexaenoic acid (22 : 6) and 7,10,13,17-docosatetraenoic acid (22 : 4). NEFA purity was checked by thin-layer chromatography (benzene/methanol/acetic acid, 96 : 4 : 1, v/v). Stock solutions of NEFAs (1 mg/ml) were prepared, using hexane for unsaturated fatty acids and chloroform for saturated fatty acids.

Blood samples

Blood samples were obtained from the umbilical cord of 3–4-month-old human fetuses (after therapeutic abortion) and hepatoma patients. Rats were made hepatotoxic by injection of carbon tetrachloride (CCl_4): 5-week-old Sprague Dawley rats (Charles River, France) were injected, i.p., with 100 μl CCl_4 in 500 μl sesame oil per 100 g body weight. Fetal rat and mouse serum was taken from 19-day rat embryos and 18-day mouse embryos. All blood samples were centrifuged immediately after collection and the serum was stored at −80 °C until used.

Purification of AFPs

AFPs were purified from fetal serum samples by affinity chromatography using specific antisera raised in rabbit against rat and mouse AFP purified by acrylamide gel electrophoresis as previously described [10]. Anti-human monospecific AFP antibodies were a gift from Dr. Keckaert (Lille).

The IgG from these specific antisera were purified by DEAE-Trisacryl chromatography in 0.035 M Tris, 0.025 M NaCl buffer (pH 8.8). The IgG antibody fractions were dialysed against 0.05 M NaCl and coupled to CNBR-activated Sepharose 4B (Pharmacia). AFP was bound at pH 8.6 (0.1 M borate buffer, 0.5 M NaCl). Human AFP was eluted with 0.1 M sodium acetate, 3 M sodium thiocyanate, pH 4; rat and mouse AFPs were eluted with 0.2 M glycine-HCl, 0.5 M NaCl (pH 2.8). The eluted AFP solutions were brought to pH 7 with 0.1 M NaOH. All purified AFP solutions were dialysed for 48 h against distilled water (pH 7) at 4°C. The purified AFPs contained endogenous NEFAs: their concentrations were 4 mol NEFA/mol AFP (human), 5 mol NEFA/mol AFP (rat) and 7 mol NEFA/mol AFP (mouse).

These purified AFP preparations are referred to as native AFPs (N-AFP); the native purified AFPs incubated with exogenous FA are referred to as fatty acid-loaded AFP (L-AFP).

Native purified human and mouse AFPs migrated as broad bands in the α zone on 10% polyacrylamide gel electrophoresis, while rat AFP showed two distinct bands in the α zone. None of the purified AFP preparations produced immunoprecipitin lines against their respective rabbit anti-adult serum (5%) in crossed immunoelectrophoresis, and showed a single immunoprecipitin line against their respective anti-fetal sera (10%).

Antisera

Antibodies were raised in rabbit by the Vaitukaitis method [24].

Monospecific antibodies were raised against the mouse, rat and human N-AFPs, where N-AFP is the purified AFP preparation described above which con-

tains a certain amount of endogenous NEFA. Lipidated AFP (L-AFP) was prepared by incubating 50 μg rat N-AFP in normal saline solution with 500 μg of 22:6, (2 μmol 22:6/nmol AFP, in 0.2 ml) overnight. This L-AFP preparation was used to raise monospecific anti-rat L-AFP antibodies.

Four subcutaneous injections of 20 μg N-AFP or L-AFP in complete Freund's adjuvant were given at 15-day intervals. Immunoglobulins were precipitated from serum at 40% saturated ammonium sulfate and chromatographed on a DEAE-Trisacryl column (0.025 M Tris-HCl, 0.035 M NaCl (pH 8.8)). The N-AFP antibodies and L-AFP antibodies were dialysed against 0.05 M NaCl (pH 7) and stored at −20 °C.

Binding studies

Batchwise gel equilibrium (25) at 20 °C and Scatchard graphic analysis [26] were used to evaluate the association constant (K_a M^{-1}), dissociation constant ($K_d = 1/K_a$) and the apparent number of binding sites per mol of pure rat AFP. The same techniques were used in competition experiments with free fatty acids. The partition coefficient for E2 was estimated and found to be unaffected by the presence of free fatty acid. The Scatchard analysis and competition studies required a series of tests with fixed amounts of pure rat AFP (58 nM) and [^{3}H]E2 (1.8 nM) plus increasing quantities of non-radioactive identical (1.8 nM to 919 nM, E2 or E1) (Scatchard analysis) or heterologous ligand (7.6 nM to 760 nM, 22:6) (competition analysis). The fatty acid, in organic solvent, were evaporated to dryness under nitrogen, mixed and shaken with buffer overnight at 4 °C. The AFP and other reagents were then added. At the concentration used (0.13 to 13 nM 22:6/nM AFP) the solubility was 95%.

Preparation of lipidated AFP

Saturated or unsaturated FA (18:0 or 22:6) were dried under a stream of nitrogen and mixed with purified AFP for immediate testing or stirred with AFP overnight at 4 °C.

For HPLC gel filtration studies, 100 μg AFP were incubated with 10–500 μg of 22:6 or 18:0 (i.e., 0.02 to 1 μmol of FA/nmol AFP in 0.1 ml phosphate-buffered saline (PBS) 0.05 M phosphate, 0.15 M NaCl (pH 7.4)).

For immunoelectrophoresis studies, 2.5 μg AFP were incubated with 10–100 μg of 22:6 or 18:0 (i.e., 1–10 μmol FA/nmol AFP) in 50 μl of Laurell immunoelectrophoresis buffer 0.06 M Tris, 0.02 M barbital lactate calcium (pH 8.6).

Unbound FA were separated from bound FA by HPLC gel filtration or with a charcoal-dextran suspension (0.5/0.05 g per 100 ml).

Fatty acid measurements

Fatty acids were extracted from AFP by the method of Chen [27] and from sera as described [28]. They were quantified by gas liquid chromatography [28].

Protein determination

Protein concentrations were measured by the method of Lowry et al. [29].

HPLC and UV spectral studies

Samples were applied to a TSK G3000 SW gel filtration column (7.5 × 600 mm) connected to an LKB 2150 HPLC pump and eluted with PBS at a flow rate of 1 ml/min. Gel filtration separated the fatty acids-AFP complexes from unbound fatty acids. Protein was monitored with an LKB 2140 multiwavelength detector (190–370 nm); the UV spectra of the eluates were integrated at 1-s intervals and stored in a IBM-XT computer connected to the detector. Data compilation was performed with the LKB Wavescan program.

The spectrum of each preparation was the average of all the spectra obtained during the elution of the protein peak.

Serological methods

Native purified AFP samples and the AFP contents of a number of human biological fluids were assayed in the absence or presence of different concentrations of fatty acids by four immunological methods:

(1) Laurell rocket immunoelectrophoresis was performed as described [30].

(2) Rocket line electrophoresis was performed using the method described by Kroll [31]. N-AFP (0.5 μg) or L-AFP (2.5 μg) was mixed in an agarose gel strip (1 cm × 9 cm × 1.2 mm). A series of samples (5 μl) of purified AFP, L-AFP and CCl_4-treated rat serum was placed in wells in the contact gel close to the N-AFP or L-AFP gel strip. The antigens were then run into the agarose gel (8 cm × 9 cm × 1 mm) containing monospecific antiserum (1% anti-N-AFP or 1% anti-L-AFP). The deflection of the AFP precipitin line by the different samples were used to study the cross-reactivity and the amounts of different AFP preparations.

Immunoelectrophoresis was performed at 2 v/cm for 18 h in the Laurell buffer. The gels were dried and stained with Coomassie blue.

(3) Crossed immunoelectrophoresis was performed using the Clark and Freeman modification [32]. Dimension 1: sample proteins (5 μl: 250 ng) were electrophoresed in 1% agarose gels at 8 v/cm, using Laurell buffer at 14 °C. Dimension 2: was run overnight with the same buffer at 14 °C at 2 v/cm into 1% agarose gel containing 1% antibody. The gels were dried and stained with Coomassie blue.

(4) Immunoassay (RIA-ELISA). Human AFP was quantified with a RIA kit from Abbot Laboratories or CEA (France), and with an ELISA kit from

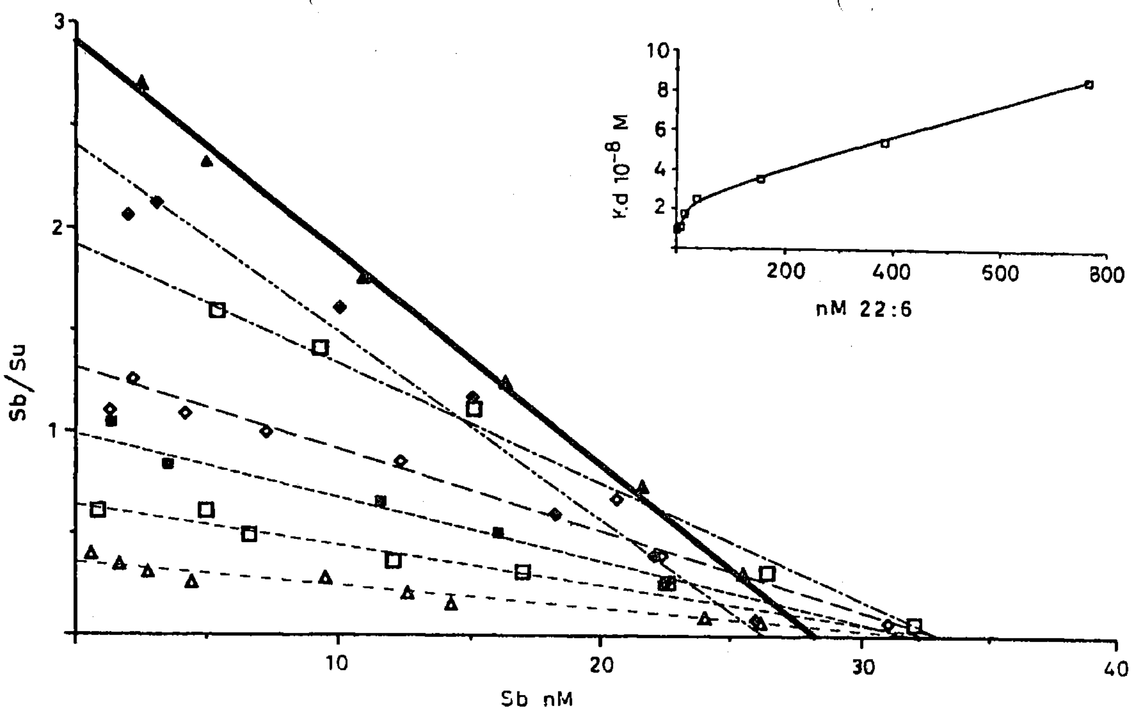

Fig. 1. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP in the presence of docosahexaenoic acid (22:6). 6 μg (58 nM) of purified AFP were incubated 1 h at 20°C with various concentration of [^{3}H]E2 (1.8–919 nM) in the absence ———▲) or presence of 7.6 nM (— — —◆), 15.2 nM (-—-—-□), 38 nM (— — —◇), 152 nM (······■), 380 nM -----□), 760 nM (— — —△) of 22:6. Steroid binding was determined by gel equilibration. The straight lines were obtained by linear regression analysis. Sb, bound steroid. Su, unbound steroid. Inset: the apparent equilibrium dissociation constant K_d was plotted against 22:6 concentration.

Boehringer-Mannheim, based on monoclonal anti-human AFP antibodies.

Results

Effects of polyunsaturated fatty acids on estradiol binding purified rat AFP

The binding parameters, association (K_a) and dissociation (K_d) constants and number of binding sites (n), were determined at equilibrium by Scatchard analysis on purified AFP with [^{3}H]E2 in the absence and presence of polyunsaturated FA (22:6).

The binding of E2 was analysed as a function of increasing 22:6 concentration. A low concentration of 22:6 (0.8 10^{-8} M) reduced E2 binding with no change in association constant, but with a slight reduction in

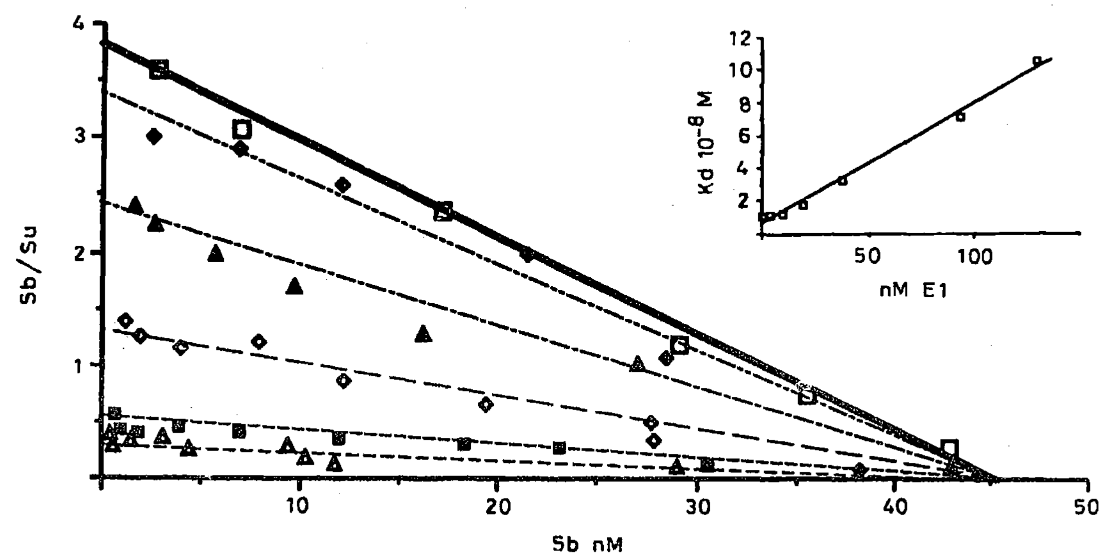

Fig. 2. Scatchard analysis of [^{3}H]E2 binding to purified rat AFP. 6 μg (58 nM) of purified AFP was incubated 1 h at 20°C with [^{3}H]E2 (1.8–919 nM) in the absence (———□) or presence of 9 nM — — —◆), 18 nM (-—-—-◆), 36 nM (— — —◇), 90 nM (-----■), 130 nM (-----△) of non-radioactive estrone (E1). Steroid binding was determined by gel equilibrium. Inset: the apparent equilibrium dissociation constant K_d was plotted against E1 concentration.

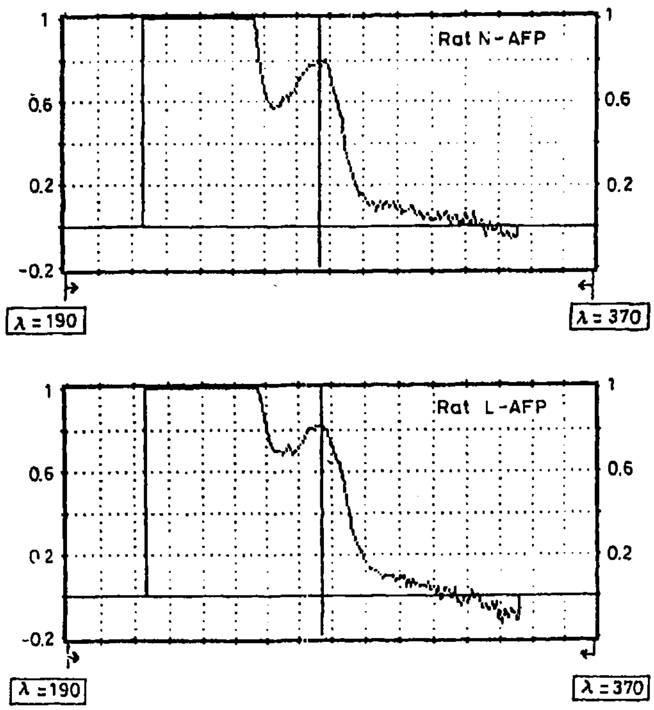

Fig. 3. UV spectra of rat N-AFP and L-AFP. 100 μg of N-AFP or AFP incubated overnight at 4°C in the presence of 0.2 μmol 22:6/nmol AFP (L-AFP) were injected and HPLC chromatographed on a TSKG 3000 SW column in 0.15 M NaCl, 0.05 M phosphate buffer (pH 7.4). Absorption from 190 to 370 nm. The spectrum was the average of all the spectra obtained during elution of the peak. Wavelength of the cursor location is 276 nm.

the number of binding sites ($n = 0.5$ to $n = 0.4$). Higher 22:6 concentrations ($> 1.6 \cdot 10^{-8}$ M) produced a significant dose-dependent decrease in the K_a and a slight increase in the number of binding sites ($n = 0.6$).

When K_d, the apparent equilibrium constant of dissociation for the steroid, was plotted against UFA concentration (inset Fig. 1), the K_d values for E2 increased non-linearly with 22 : 6 concentration. However, Scatchard analysis of E2 binding to AFP in the presence of increasing amounts of unlabelled estrone or estradiol indicated that the K_d for this interaction varied linearly with both increasing concentration of E1 (Fig. 2) and E2 (data not shown).

Effect of fatty acids on AFP spectral properties

The elution profile from HPLC gel filtration column and the UV spectral analysis of the rat N-AFP and L-AFP indicated that both AFP preparations were eluted with the same retention time (14 min and 17 s in our experimental conditions) and showed the same maximum absorbance wavelength ($A_{max} = 276$ nm). However, the absorbance minimum was less pronounced for the L-AFP than for N-AFP (Fig. 3). A plot of the logarithm of the ratio of maximum absorbance at 276 nm over the absorbance at a given wavelength (log $A_{276\ nm}/A_{\lambda nm}$) against wavelength ($\lambda = 245$ to 276 nm), showed that the absorbance minimum (which was 253–254 nm for N-AFP) was red-shifted and broadened to 262 nm for L-AFP (0.2 μmol 22:6/nmol rat AFP) (Fig. 4A). The extent of this shift appears to be FA-dose-dependent. The value of the ratio log $A_{276\ nm}/A_{254\ nm}$ was lower for L-AFP (0.08) than for N-AFP (0.15).

There were similar changes in the absorption spectra of fatty acid-loaded human AFP (L-AFPh) (Fig. 4B). The value of log $A_{276\ nm}/A_{254\ nm}$ dropped in the same dose-dependent manner as for the rat, from 0.15 (N-AFPh) to 0.08 (in the presence of 0.2 μmol 22:6/nmol AFP) and 0.04 (in the presence of 1 μmol 22:6/nmol AFPh). The wavelength 247 nm was taken as the pivotal wavelength at which the ratio plot values for N-AFP were positive and those for L-AFP were negative, for both rat and human AFP.

No spectral changes were seen when either human or rodent AFPs were incubated with saturated fatty acids.

No spectral changes occurred when transferrine, a protein which is not known to bind FA, was incubated

TABLE I

Qualitative and quantitative analysis of non-esterified fatty acids and endogenous content of different biological fluids

NEFA classes	Cord serum		Hepatoma serum		Fetal serum	
Total NEFAs μM:	188		1590		1691	
	(μM)	(%)	(μM)	(%)	(μM)	(%)
Saturated 14:0	6.5		25.6		–	
16:0	45.6	35.2	27.8	27.25	216.4	35.2
18:0	39.4		129.3		379.6	
Unsaturated						
mono 16:1	10.9		200		151	
18:1	47.2	28.8	490	43.3	333.6	28.8
di+tri 18:2	28.8	15.3	346.5	21.8	227.5	13.5
18:3						
poly 20:4	9.9	5.25	109		130.3	
22:4	–		–	7.65	–	22.5
22:6	–		12.5		252.6	

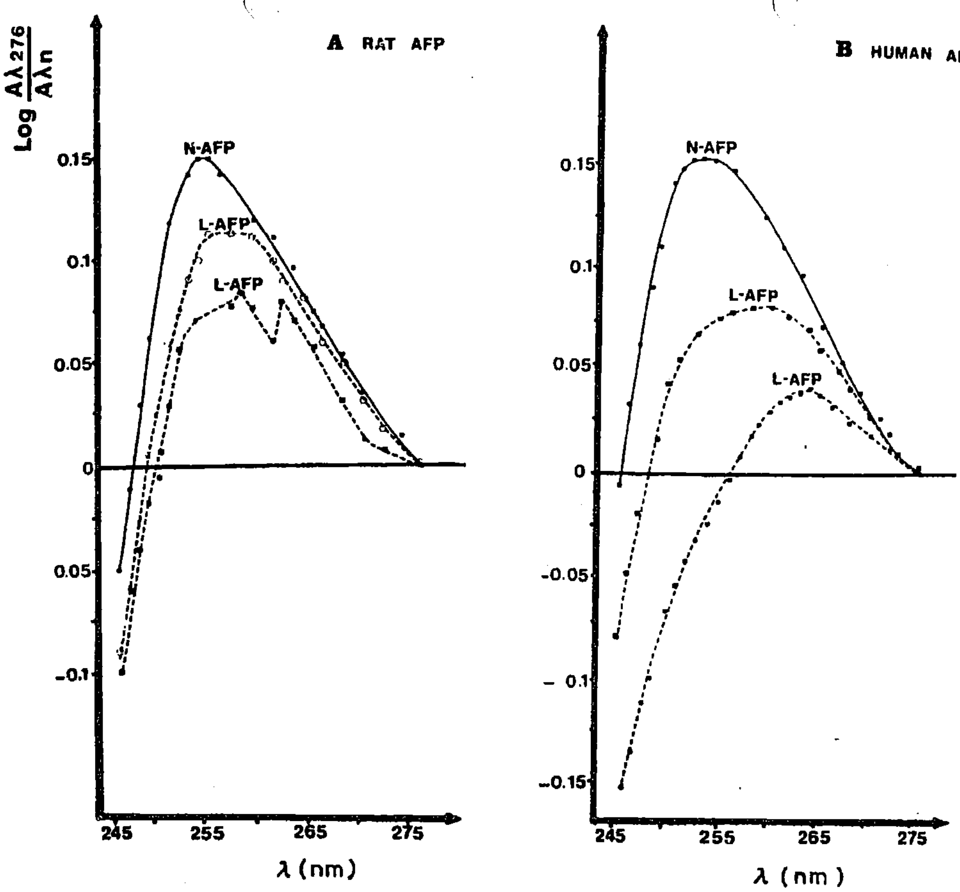

Fig. 4. Plot of the ratio log $A_{276\ nm}/A_{245\ nm\ to\ 276\ nm}$ for N-AFP and L-AFP as a function of wavelength. (A) Rat N-AFP and L-AFP. L-AFP: 100 μg rat AFP were incubated with 50 μg 22:6 (0.1 μmol 22:6/nmol AFP) (○— — —○) and 100 μg 22:6 (0.2 μmol 22:6/nmol AFP) (■— — —■) in 0.1 ml phosphate buffer, overnight at 4°C. (B) Human N-AFP and L-AFP. L-AFP: 100 μg human AFP were incubated with 100 μg 22:6 (0.2 μmol 22:6/nmol AFP (■— — —■) and 500 μg 22:6 (1 μmol 22:6/nmol AFP) (●— — —●) in 0.1 ml phosphate buffer overnight at 4°C.

with increasing concentrations of unsaturated fatty acids (data not shown).

Effects of fatty acids on the immunological properties of rodent and human AFP

The immunoreactivities of N-AFP and L-AFP were first studied with specific polyclonal anti-N-AFP antibodies.

The immunoreactivities of purified rodent and human AFPs were not changed by the presence of saturated fatty acids (e.g., stearic acid), but incubation with unsaturated fatty acids caused major changes in the immunological behaviour of AFP. The unsaturated fatty acid-loaded AFP (L-AFP) from mouse, rat and human migrated slightly faster than the corresponding N-AFPs and were considerably less immunoreactive towards specific polyclonal anti N-AFP antibodies (Fig. 5).

Rocket-line immunoelectrophoresis revealed a loss of L-AFP immunoreactivity with anti-N-AFP antibodies. There was a clear precipitin line between the rat N-AFP of the intermediate gel insert and the N-AFP antiserum in the upper gel (Fig. 6A). The line was deflected to form a well-focused 'rocket' with N-AFP (1st and 2nd wells), but the deflection was very much smaller for the same quantity of L-AFP (3rd and 4th wells).

However, when rat L-AFP was placed in the intermediate gel strip (Fig. 6B), the precipitin line formed with the anti-N-AFP antibody was fuzzy, indicating poor recognition of the L-AFP by polyclonal anti-N-AFP.

The L-AFP rocket was totally fused with the L-AFP line, while the N-AFP rocket showed additional spurs. Thus, some antigenic site(s) were common between N-AFP and L-AFP, but N-AFP had more epitopes than did L-AFP.

When the AFP in a pathological serum (CCl_4-treated rat serum) was tested against an anti-N-AFP antibody (Fig. 6), this AFP deflected the L-AFP line (Fig. 6B) more than the N-AFP line (Fig. 6A). The total fusion of rockets formed by the AFP from CCl_4-treated rat serum with the L-AFP line indicated that it was immunological very similar to L-AFP. Thus the AFP from this pathological serum seems to be different from rat embryo serum AFP.

The differential behaviour of N-AFP and L-AFP led us to prepare specific polyclonal anti-L-AFP antibodies. The immunoreactivity of these antibodies towards both

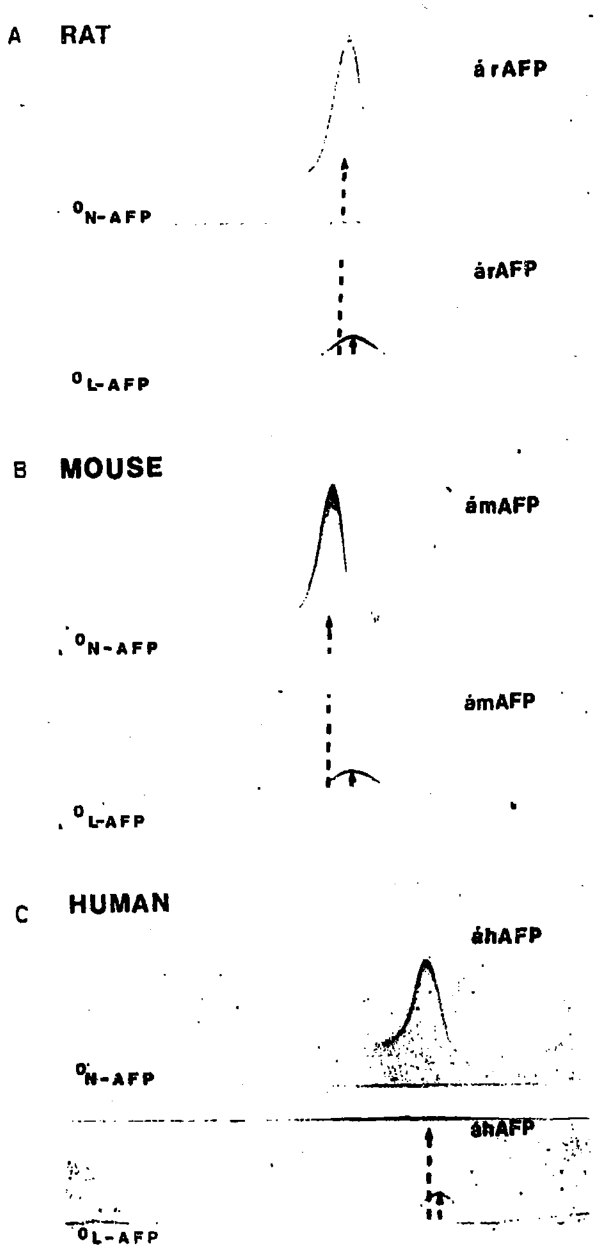

Fig. 5. Crossed immunoelectrophoresis stained with Coomassie blue, with purified rat (A), mouse (B) or human (C) AFP (250 ng) and L-AFPs (250 ng). The L-AFPs were prepared by preincubating AFPs overnight with 22:6 (25 μmol 22:6/nmol AFP). Antiserum 1% = rabbit anti-rat N-AFP (àrAFP), anti-mouse N-AFP (àmAFP), anti-human N-AFP (àhAFP).

native and lipidated AFP preparations were then studied (Fig. 7A, B and C). Rocket-line immunoelectrophoresis with L-AFP in the intermediate gel strip (Fig. 7A) showed a distinct precipitin line with anti-L-AFP antibodies which was clearly deflected by L-AFP, but only fuzzily deflected by N-AFP. By contrast, rocket line immunoelectrophoresis with N-AFP included in the intermediate gel strip (Fig. 7B) showed a fuzzy precipitin line for N-AFP. The L-AFP placed in the wells produced a very small deflection of the fuzzy N-AFP precipitin line, together with an additional clear rocket under the fuzzy precipitin line. These results suggest that the lower part of gel was depleted of N-AFP antibodies and that the well-focused rocket observed with L-AFP is due to the specific recognition of this holoform of AFP by conformation specific-sites of 'monoclonal subclasses' of the polyclonal antibodies raised against L-AFP.

This hypothesis was checked by removing N-AFP antibodies from the specific L-AFP antibodies with a preliminary migration of N-AFP included in the intermediate gel for 8 h before placing N-AFP or L-AFP in the well (Fig. 7C). This treatment resulted in the production of a rocket with only the L-AFP from (wells 2 and 3), while the N-AFP form was no longer seen (wells 0 and 1).

Such results and changes in AFP immunoreactivity can lead to apparent differences in AFP quantitation as a function of the free fatty acid environment. This variation depends largely on the biological fluid or the pathophysiological situation analyzed (Table I). Previous studies, using Laurell immunoelectrophoresis and anti-rodent or anti-human polyclonal antibodies, showed that the quantitation of AFP incubated with polyunsaturated fatty acid was reduced or difficult to determine because of the fuzzy appearance of the rockets. Similar results were obtained even when the AFP-fatty acid mixture had been treated with charcoal-Dextran suspension to remove unbound lipid.

Purified human AFP was quantified by RIA (Abbot and CEA) and ELISA using monoclonal antibody in the presence of polyunsaturated fatty acid (Fig. 8). There was a progressive loss of immunoreactivity with increasing polyunsaturated fatty acid (22:6) concentration. The apparent concentration of purified AFP dropped sharply up to a concentration of 0.2 μmol 22:6 per ml in the incubation mixture. Higher FA concentrations produced no further significant modification of AFP immunoreactivity (Fig. 8A). Human AFP in different biological fluids (cord serum, hepatoma serum and fetal serum) was assayed in the presence and absence of increasing concentrations of exogenous polyunsaturated fatty acid (22:6) (Fig. 8B).

The estimations of AFPs in fetal and hepatoma serum were FA dose-dependent. The estimated AFP concentration in the presence of 3 μmol FA per ml was only 10% (fetal serum) or 50% (hepatoma serum) of the control value measured in the absence of exogenous FA. The estimation of AFP in cord blood was the least affected by exogenous FA (95% control at all the FA concentrations tested). These results may be correlated with the level of fatty acid in each biological fluid (Table I). The lowest level of fatty acid was found in cord serum, which also had the lowest relative percentage of polyunsaturated fatty acid, while the fetal serum had the highest fatty acid content and the highest relative percentage of polyunsaturated fatty acid.

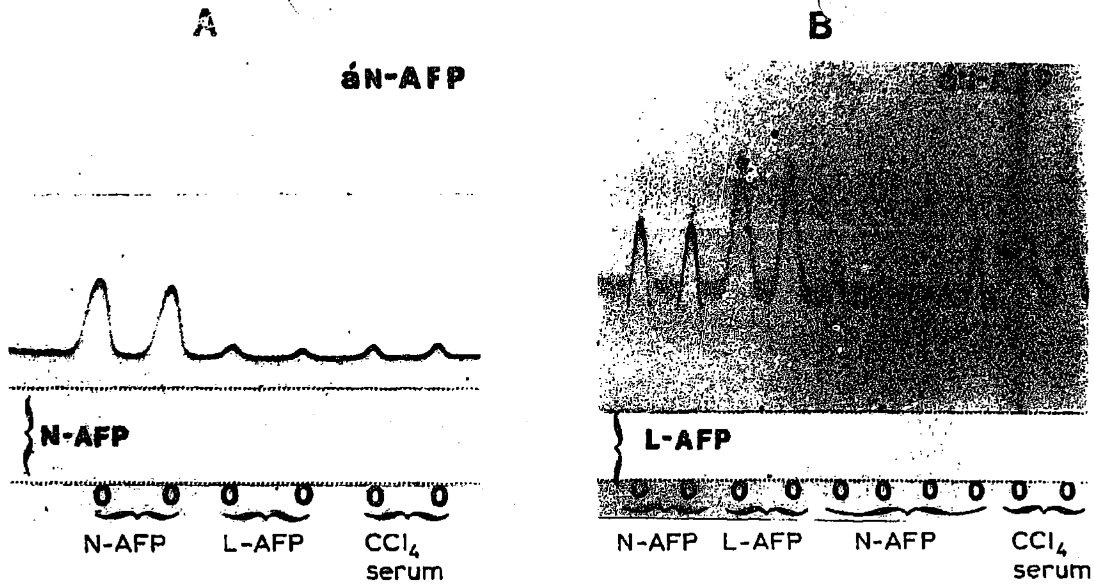

Fig. 6. Rocket-line immunoelectrophoresis of rat N-AFP (A) and L-AFP (B) against anti-rat N-AFP antibodies (àN-AFP). (A) N-AFP (500 ng) was included in the intermediate gel strip. The sample wells contained: N-AFP (50 ng), L-AFP (50 ng), 5 μl of CCl_4-treated-rat serum. The L-AFP was prepared by preincubating AFP with 22:6 (5 μmol 22:6/nmol AFP). 1% anti-rat N-AFP antiserum (àN-AFP). (B) L-AFP (2.5 μg) was included in the intermediate gel strip. The sample wells contained: N-AFP (100 ng), L-AFP (100 ng), N-AFP used as standards (37, 25, 12 and 50 ng), 5 μl of CCl_4-treated rat serum. Antiserum: anti-N-AFP antibodies (àN-AFP) (1%).

Discussion

AFP has been described as a flexible monomeric three-domain structure with hydrophilic external surfaces and large, deep hydrophobic pockets [33]. The present binding, spectral and immunochemical studies show that the high-affinity ligands, unsaturated fatty acids, can induce conformational changes in AFP.

Previous studies have shown that the binding of estrogen to rodent AFP is not affected by saturated fatty acid but is inhibited by unsaturated fatty acid in a dose-dependent manner and as a function of their degree of unsaturation [5]. The present binding study shows that the parameters, K_a and n, for E2 binding to rat AFP are modified in the presence of increasing concentrations of unsaturated fatty acid. The K_d varied non-linearly as a function of increasing unsaturated fatty acid concentration and this relationship may be a reflection of 22:6-induced changes in AFP conformation. This results might also indicate that there are

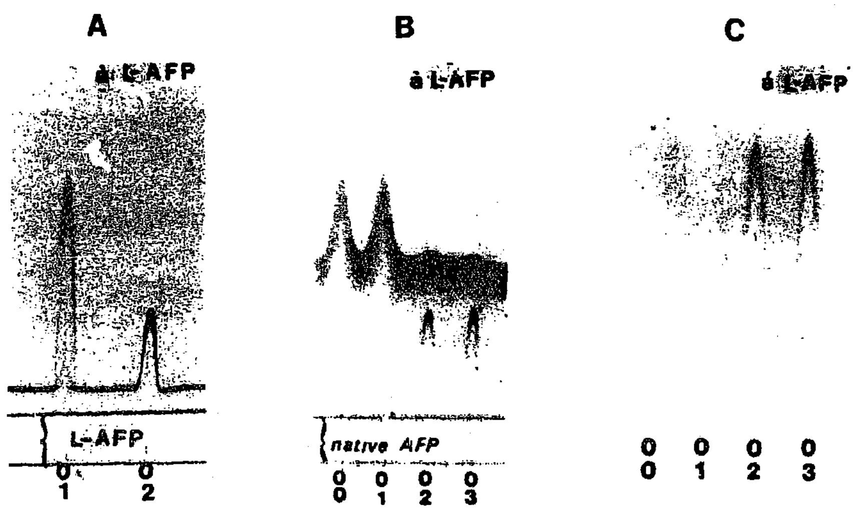

Fig. 7. Immunoreactivity of rat N-AFP and L-AFP against anti-rat L-AFP antibodies. Rocket-line immunoelectrophoresis studies. (A) L-AFP (10 μg) was included in the intermediate gel. The L-AFP was prepared by preincubating AFP overnight with 22:6. The sample wells contained: N-AFP (250 ng) (well 1), L-AFP (250 ng) (well 2). (B) N-AFP (10 μg) was included in the intermediate gel. The sample wells contained N-AFP (250 ng) (wells 0 and 1) L-AFP (250 ng) (wells 2 and 3). (C) N-AFP antibodies were removed from the specific L-AFP antibodies by a preliminary migration of N-AFP (20 μg) included in the intermediate gel for 8 h. This latter was subsequently removed before planing N-AFP (500 ng) (wells 0 and 1) or L-AFP (500 ng) (wells 1 and 2) in the wells.

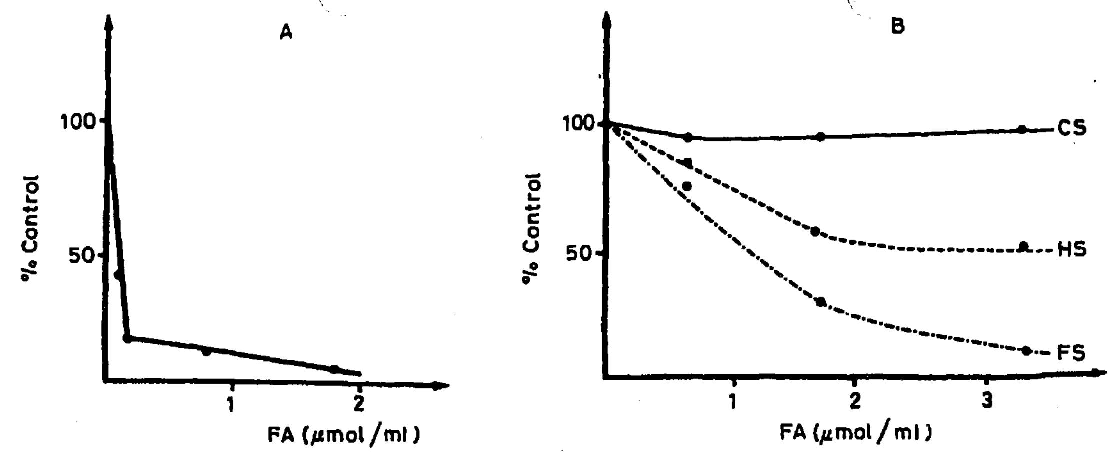

Fig. 8. (A) Radioimmunoassay of AFP purified from human fetal serum, in the presence of increasing concentrations of 22:6. RIA was performed with the Abbot kit. Results are expressed as percent of the control (100%) measured without 22:6. The concentration of AFP control was 0.11 nmol/ml. (B) Radioimmunoassay of human AFP in cord serum (CS), hepatoma serum (HS) and fetal serum (FS) in the presence of increasing concentrations of exogenous 22:6. RIA was performed with the Abbot Kit. The reaction mixtures contained the appropriate concentrations of unsaturated FA plus an aliquot of serum equivalent to approx. 10 μg AFP. The results were expressed as % of their respective controls. The concentration of AFP in different sera before incubation with 22:6 was taken as 100%.

several fatty acid binding sites on rodent AFP ($n = 4$ for 20:4, data not shown) with two classes of affinity binding site [7]. Thus, about 7.6 nM 22:6 would saturate the sites that do not affect the K_d of E2 binding (non-competitive binding), while over a range of 38–760 nM 22:6, the K_d of E2 varied linearly, suggesting that as with tryptophane methyl ester [34], 22:6 induces competitive inhibition. The K_d for the E2-AFP complex changed linearly with increasing amounts of either E1 or E2, suggesting that, there is a reciprocal competition between the steroid hormones. Thus unsaturated fatty acids appear to affect the conformation of AFP, specifically influencing its estrogen-binding activity. Similar effects of unsaturated fatty acid have been reported for corticosteroid-binding globulin (CBG) and anti-estrogen sites [12,35].

Analysis of multi-wavelength HPLC output profiles showed that the absorption minima of rodent and human L-AFPs incubated with unsaturated fatty acid were red-shifted and broadened in a dose-dependent manner (5 nm to 10 nm) and that the absorbance minimum was greater for L-AFP than for N-AFP. The changes in the multiwavelength ratio plots for rat and human AFP with fatty acid concentration seem to be very similar. They were not changed by incubation with saturated fatty acid. These variations in the UV spectral properties of rodent and human AFP with the unsaturated fatty acid environment may reflect the absorption of the unsaturated fatty acid bound to AFP. They may also provide indices of unsaturated fatty acid-induced conformational changes in AFP. Similarly, human AFP conformational states with characteristic changes of the accessibility of the hydrophobic sites has been demonstrated as a function of acid or alkaline environments [33].

The immunological results may also provide additional evidence for FA-mediated conformational changes of AFP. High-titer-specific polyclonal antibodies against N-AFP and L-AFP preparations were used to check the hypothesis that there are several conformational states corresponding to different forms of lipidated AFP (holoforms).

N-AFP antibodies recognized fewer epitopes on L-AFP than on N-AFP (Fig. 6), indicating that either FA directly masks some epitopes of N-AFP, giving rise to a partial identity between N-AFP and L-AFP, or that FA binding is associated with a conformational change in N-AFP to cause rearrangement of surface antigen sites and bring about quantitative and qualitative alteration in the reaction with antibody. The addition of FA to N-AFP without prior incubation did not disturb the immunodetection of N-AFP, and charcoal-dextran treatment to remove the unbound FA did not change the L-AFP holoform recognition by anti-N-AFP antibodies. Thus, the perturbation of the immunoprecipitation process was not due to the FA alone; it is more likely that this is caused by a conformational change of AFP in the presence of FA. L-AFP antibodies reacted poorly with N-AFP in the rocket-line system, while reacting well with the L-AFP holoform.

The N-AFP and L-AFP injected as immunogens probably correspond to equilibrium mixtures of different, more or less lipidated, states of AFP. Such equilibrium states could explain why the L-AFP was slightly recognized by anti-N-AFP antibodies and, why N-AFP was slightly recognized by anti-L-AFP. Line immunoelectrophoresis and the use of specific polyclonal antibodies offer an excellent screening procedure for detecting, characterizing and isolating the class of antibodies directed against each specific conformational state of a

protein. Thus, the absorption of the polyclonal L-AFP antibodies in situ by N-AFP antigen in the gel strip shows that it is possible to isolate classes of antibodies which react specifically with the L-AFP form (Fig. 7C). These results provide an additional argument for the being conformational transition states of AFP which are dependent on its hydrophobic environment.

The biological environment of the AFP, especially the NEFAs which varies greatly during ontogenesis [36], oncogenesis and in pathological situations, could confer particular conformational states on this oncofetal protein. This is emphasized by the immunodetection of AFP in different biological fluids. The immunopattern of AFP from CCl_4-treated rat serum was close to that of L-AFP holoforms, suggesting that the conformation of this pathological AFP is different from that of fetal AFP. The AFP immunopattern in CCl_4-treated rats may be correlated with the fact that the serum of these rats had a relatively high percentage of polyunsaturated FA (44%) and low AFP concentrations (μg/ml) compared to fetal serum (23% polyunsaturated FA, mg/ml AFP).

The apparent values of AFP obtained by radioimmuno-assays of various biological fluids were modified differentially by exogenous unsaturated FA. This could be explained by the existence of different conformational states of the protein more or less sensitive to the effect of exogenous FA. The difference in sensitivity to FA of the various AFP could be related to their NEFA environment in each biological fluid studied. Indeed, the free fatty level in cord blood serum is much lower (10-fold) than that of hepatoma or fetal serum. Moreover, the relative percentage of polyunsaturated fatty acid was very low in cord blood serum (5%) comparatively to hepatoma serum (8%) and fetal serum (23%). However, these results may also be due to other factors, such as the presence of different isoforms of AFP in each of the sera, with the combination of forms found in the cord blood serum being much less sensitive to fatty acids. The original choice of AFP cord serum as the international standard could be explained by such results [37]. Some of the problems encountered in the measurement of AFP may, in some pathophysiological situation, be linked to these differences in FA sensitivity [38].

These FA-induced changes in the spectral, electrophoretic and immunclogical behaviour of AFP and in its functional properties (binding) tend to indicate that this oncofetal protein has an enormous adaptative and interactive capacity towards the environmental changes which occur during normal and pathological development.

Hence endogenous or exogenous (nutritional) NEFAs may modify the three-dimensional structure of AFP, with the reciprocal interaction of saturated and unsaturated fatty acids in the immediate AFP environment contributing to the formation of transient forms of AFP (holoforms). Some of these holoforms could interact with specific membrane receptors and become internalized [21,39]. The presence of multiple holoforms may thus explain the inhibitory or stimulatory effect of AFP on the response of immune cells [14–23] or estrogen target cells [20,40]. These transient forms could comprise one of the keys to the multiple functional potentiality of the AFP.

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